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14. ABSTRACT Tumor-induced myeloid-derived suppressor cells (MDSC) contribute to immune suppression in tumor-bearing individuals and are a major obstacle to effective immunotherapy. Reactive oxygen species (ROS) are one of the mechanisms used by MDSC to suppress T cell activation. Although ROS are toxic to most cells, MDSC are not negatively impacted by their production of ROS. Nuclear factor erythroid derived 2-like 2 (Nrf2) is a transcription factor that regulates a battery of genes which attenuate oxidative stress and therefore we hypothesized that MDSC resistance to ROS may be due to their up-regulation of Nrf2. To test this hypothesis, we utilized BALB/c and C57BL/6 mice bearing 4T1 mammary carcinoma and MC38 colon carcinoma, respectively. MDSC from the peripheral blood of Nrf2 ^{-/-} mice with 4T1-tumors were more oxidatively stressed and apoptotic, produced less H ₂ O ₂ , and were less suppressive than MDSC from Nrf2 ^{+/+} mice, indicating that Nrf2 sustains MDSC survival and suppressive activity. Primary tumors and levels of MDSC were similar in Nrf2 ^{-/-} and Nrf2 ^{+/+} mice, but Nrf2 ^{-/-} mice survived longer. Since Nrf2 ^{-/-} MDSC were more apoptotic, but Nrf2 ^{-/-} and Nrf2 ^{+/+} tumor-bearing mice had similar levels of MDSC, we hypothesized that MDSC differentiate more rapidly from Nrf2 ^{-/-} than from Nrf2 ^{+/+} progenitor cells. This hypothesis was confirmed because Nrf2 ^{-/-} bone marrow cells cultured with IL-6 and GM-CSF, produced more MDSC than similar cultures of Nrf2 ^{+/+} progenitor cells. These data demonstrate that Nrf2 facilitates tumor progression by increasing MDSC-mediated suppression and by delaying MDSC turnover, and identify Nrf2 as a potential therapeutic target for reducing tumor-induced immune suppression and enhancing cancer immunotherapy.					
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Table of Contents

	Page
Introduction.....	4
Key Words.....	5
Project Summary.....	5
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	9
Bibliography.....	9
References.....	11
Supporting Data.....	12

Introduction

Immune suppression is a major obstacle to breast cancer immunotherapy. A primary reason that immunotherapy is not effective is due Myeloid-Derived Suppressor Cells (MDSC). MDSC are a heterogeneous population of immature myeloid cells that accumulate in the blood, secondary lymphoid organs, and in primary and metastatic tumors in tumor-bearing individuals. MDSC are characterized by the surface markers Gr1 and CD11b in mice, and CD33 and CD11b in humans [1-3]. Granulocytic MDSC are characterized as CD11b⁺ Ly6G⁺/Ly6C⁻ in mice (HLA-DR⁻ CD11b⁺ CD33⁺ CD15⁺ in humans), while monocytic MDSC in mice are CD11b⁺ Ly6G⁻/Ly6C⁺ (HLA-DR⁻ CD11b⁺ CD33⁺ CD14⁺ in humans). A variety of endogenous factors including vascular endothelial growth factor (VEGF) [4], prostaglandin E2 (PGE2) [5], IL-1 β [6, 7], IL-6 [8], S100A8/A9 [9, 10], the complement component C5a [11], and endotoxin [12] induce the accumulation of MDSC. MDSC block adaptive anti-tumor immunity by inhibiting the activation of CD4⁺ and CD8⁺ T lymphocytes [2, 13, 14]. MDSC also produce IL-10, which polarize macrophages to a tumor prototyping phenotype [15, 16]. A primary mechanism of MDSC-mediated suppression of T cells is by MDSC production of short-lived oxidants such as reactive oxygen species (ROS), nitric oxide, and peroxynitrite [17]. These reactive oxidizing agents are vital for T cell repression and for maintaining the inflammatory tumor microenvironment [18]. However, MDSC survive despite their high levels of these non-discriminatory toxic radicals. I hypothesize that MDSC withstand these oxidizing agents due to the transcriptional regulator Nrf2. NF-E2 related factor 2 (Nrf2) is stabilized by the same factors that induce MDSC accumulation and suppression, and when activated, induces the expression of protective and survival genes for antioxidant responses, phase II detoxification enzymes, and a plethora of other genes. These genes are grouped based on function and include genes for detoxification, antioxidant response, transcription, growth, defense and inflammatory response, signaling, and others [19, 20]. Nrf2 regulates genes controlled by the anti-oxidant response element (ARE) [21, 22] that are responsible for antioxidant responses, including glutathione synthesis genes such as GCL (Glutamate-Cysteine Ligase), and cystine transport genes x_cT and 4F2 [19, 23]. Cystine transport and Nrf2 may contribute to MDSC survival. We have previously shown that MDSC sequester cystine [24]. This sequestration may facilitate MDSC resistance to toxic radicals since importation of cystine (via the x_c⁻ cystine/glutamate antiporter) and its reduction to cysteine are rate-limiting for the synthesis of the antioxidant glutathione (GSH) in MDSC. Nrf2 is a major transcriptional regulator of x_c⁻ and GSH synthesis genes [19, 20, 23]. Nrf2 is activated by the same oxidative radicals that MDSC use to facilitate immune suppression. Nrf2 protects cells against inflammation and is stabilized in response to inflammation, hypoxia, and other factors that are known inducers of MDSC. Since Nrf2 regulates antioxidant response and apoptosis, I hypothesize that Nrf2 regulates MDSC survival by protecting MDSC from oxidative stress. To test this hypothesis, I will be utilizing tumor-bearing Nrf2 deficient and Nrf2^{+/+} mice and comparing MDSC function and apoptotic rate in addition to monitoring these mice for survival and metastatic disease.

Aim 1: Determine if Nrf2 regulates MDSC survival by testing Nrf2^{-/-} and Nrf2^{+/+} MDSC for apoptotic marker expression *in vivo* and the rate of apoptosis *in vitro*.

Aim 2: Determine if Nrf2 regulates tumor-bearer survival and MDSC suppressive activity.

Aim 3: Determine if blocking cystine transport into MDSC while providing T cells with cysteine is a therapy for reducing MDSC-mediated immune suppression and delaying the growth of primary and metastatic mammary carcinomas.

Completion of these aims will determine if Nrf2 is a critical regulator of MDSC function and survival. New insight into Nrf2 modulating MDSC activity will provide future avenues for targeting MDSC as an adjuvant to cancer immunotherapy.

Keywords

7AAD: 7-Aminoactinomycin D

DCFDA: Dichlorofluorescein diacetate

GM-CSF: Granulocyte marker colony stimulating factor

GSH: Glutathione

IL-6: Interleukin 6

IL-10: Interleukin 10

ROS: Reactive Oxygen Species

MDSC: Myeloid-Derived Suppressor Cells

PI: Propidium iodide

x_C⁻: System x_C⁻

xCT: Cystine/Glutamate Antiporter

Project Summary

Aim 1- In Progress

MDSC are functional immune suppressors despite their exposure to constant oxidative stress. Blood MDSC have high levels of ROS and tumor-infiltrating MDSC produce even more ROS (Previous data). Tumor-infiltrating MDSC are also exposed to even more oxidative stress due to the poorly vascularized and hypoxic tumor microenvironment and by ROS produced directly by tumor cells. Despite high levels of oxidative stress, MDSC are functionally suppressive and do not apoptose. *I hypothesize that MDSC resist apoptosis from oxidative stress by the activity of Nrf2.* If MDSC lack Nrf2, then they would be more susceptible to apoptosis from oxidative stress.

Nrf2 decreases MDSC oxidative stress in vitro and in vivo. To determine if Nrf2 regulates MDSC oxidative stress in vitro, we examined oxidative stress in MDSC derived from Nrf2^{+/+} and Nrf2^{-/-} bone marrow progenitor cells by DCFDA staining (Figure 1). Nrf2^{+/+} MDSC derived from bone marrow progenitor cells were less oxidatively stressed than Nrf2^{-/-} MDSC (Figure 1A). To determine if Nrf2 regulates MDSC oxidative stress in vivo, we analyzed MDSC from the peripheral blood of BALB/c Nrf2^{+/+} and Nrf2^{-/-} mice bearing syngeneic 4T1 mammary carcinoma for oxidative stress. Tumor-induced Nrf2^{+/+} MDSC were also less oxidatively stressed than Nrf2^{-/-} MDSC (Figure 1B). These data indicate that Nrf2 protects MDSC from oxidative stress in vitro and in vivo.

Nrf2 protects MDSC from apoptosis. Since Nrf2 protects MDSC from oxidative stress, we determined if Nrf2 guards MDSC from apoptosis. Bone marrow progenitor cell-derived and 4T1 tumor-induced MDSC were analyzed for apoptosis by Annexin V staining (Figure 2). MDSC derived from Nrf2^{+/+} bone marrow progenitor cells were less apoptotic than Nrf2^{-/-} MDSC (Figure 2A). Tumor-induced Nrf2^{+/+} MDSC were also less apoptotic than Nrf2^{-/-} MDSC (Figure 2B). These data indicate that Nrf2 protects MDSC from apoptosis in vitro and in vivo.

Nrf2 does not impact the level of MDSC in tumor-bearing animals. Since Nrf2 protects MDSC from oxidative stress and apoptosis, I hypothesized that there would be more MDSC in tumor-bearing Nrf2^{+/+} mice compared to Nrf2^{-/-} mice. To test this hypothesis, we determined if the percentage of MDSC in peripheral blood was different between 4T1 or MC38 tumor-bearing Nrf2^{+/+} and Nrf2^{-/-} mice (Figure 3). MDSC percentage

increased over time with increasing tumor burden in both tumor models. However, there was no difference MDSC accumulation between tumor-bearing Nrf2^{+/+} and Nrf2^{-/-} mice.

Nrf2 deficiency enhances granulocytic MDSC proliferation. Since Nrf2 protects MDSC from apoptosis, but does not affect the quantity of MDSC in tumor-bearing mice, I hypothesized that bone marrow progenitor cells differentiate into MDSC at a greater rate in Nrf2^{-/-} mice compared to Nrf2^{+/+} mice. To test this hypothesis, we analyzed MDSC subpopulations expanded from naïve Nrf2^{+/+} and Nrf2^{-/-} bone marrow progenitors (Figure 4). In both BALB/c and C57BL/6 models, there were more Nrf2^{-/-} MDSC compared to Nrf2^{+/+} MDSC (Figure 4A). These MDSC were primarily the granulocytic phenotype (Figure 4B).

Aim 2- In Progress

MDSC are functional immune suppressors and tumor-bearer survival is negatively correlated with MDSC suppressive activity [25]. Previously I had shown that MDSC from 4T1 tumor-bearing Nrf2^{+/+} mice produced more H₂O₂ and were more suppressive than MDSC from 4T1 tumor-bearing Nrf2^{-/-} mice. 4T1 metastasis is the cause of death in 4T1 tumor-bearing animals [26] and 4T1-bearing Nrf2^{-/-} animals may live longer due to enhanced resistance to metastasis. Resistance to metastasis requires a competent immune system [27]. Since Nrf2^{-/-} MDSC are less suppressive, then there would be less immune suppression and could potentially allow for tumor-bearing Nrf2^{-/-} mice to survive longer because they are more resistant to metastatic disease compared to tumor-bearing Nrf2^{+/+} animals.

Nrf2 does not impact primary tumor growth, but decreases survival time of tumor-bearing mice. Since Nrf2^{+/+} MDSC are more suppressive and oxidatively stressed than Nrf2^{-/-} MDSC, we hypothesized that Nrf2 would enhance tumor progression and MDSC accumulation of tumor-bearing mice. However, we observed that Nrf2 did not impact primary tumor growth of 4T1 or MC38 (Figure 5A). Despite similar sizes of primary tumor and levels of MDSC, we observed that 4T1 and MC38 tumor-bearing Nrf2^{-/-} animals live longer than their Nrf2^{+/+} counterparts indicating that Nrf2 decreases survival time of tumor-bearing mice (Figure 5B).

Aim 3-Completed

Aim 3 was completed during the 2010-2011 report period.

MDSC are capable of suppressing T cells by sequestering the cystine from the local environment. Cysteine exists as the dipeptide cystine in the oxidized, extracellular environment. T cells require cysteine transport through ASC, the neutral amino acid transporter, for proliferation. They rely on macrophages and dendritic cells for their source of cysteine because they lack cystathionase and x_C⁻, the cystine/glutamate antiporter. Macrophages and dendritic cells provide cysteine for T cells by transporting cystine through x_C⁻, reduce cystine to cysteine intracellularly, and then export cysteine via ASC into the local environment for T cell utilization. MDSC lack cystathionase and the ASC transporter, and are therefore not only reliant on cystine transport through x_C⁻ as their sole source of cysteine, but are capable of sequestering cystine from the local environment, thereby depriving T cells of cysteine [24]. The x_C⁻ antiporter is composed of two subunits, xCT and 4F2 [28]. MDSC express both xCT and 4F2 subunits, and inflammation-induced MDSC have higher expression of these proteins (Figure 7). However, inflammation-induced MDSC do not transport more cystine than conventional MDSC (Figure 8).

Due to the expression patterns of x_C⁻ and ASC on MDSC, other myeloid cells, and T cells, therapeutic treatment with sulfasalazine (SASP) and *N*-acetylcysteine (NAC) was utilized on tumor-bearing animals in an attempt to selectively deprive MDSC of cysteine. SASP has been shown to inhibit the x_C⁻ transporter [29] and

NAC is an extracellularly available form of cysteine that can be transported through ASC [30]. Since MDSC rely on x_c^- as their sole source of cystine, we hypothesized that SASP would inhibit MDSC sequestration of cystine, while NAC would supplement other myeloid cells and T cells with cysteine. SASP inhibits MDSC transport of cystine, decreases intracellular GSH, and reduces MDSC viability (previously shown). To determine if SASP and NAC have a therapeutic benefit for tumor-bearing animals, groups of BABL/c mice were inoculated 4T1 cells and given I.P. injections of 5mg SASP solubilized in PBS or PBS control every 12 hours and drinking water supplemented with 0.2% NAC. Data from this experiment was not meaningful since the stress associated i.p. injection induced high levels of MDSC (data not shown). A second experiment was performed utilizing groups of 10 BALB/c mice injected with 10^5 4T1 cells. Mice were given drinking water supplemented with 515 μ M SASP and assayed weekly for tumor growth, percentage of MDSC in the blood, and when mice became moribund, for spontaneous metastatic cells in the lungs and MDSC suppressive activity. SASP treatment had no effect on primary tumor growth, MDSC percentage in the blood, spontaneous metastatic disease, or MDSC suppressive activity (previously shown). Collectively, these data suggest that SASP is a poor candidate as a treatment for tumor-bearing individuals.

Key Research Accomplishments

Training Plan

Task 1: Meet yearly with my dissertation committee to review my experimental progress in the project. **(Completed to date)**

Task 2: Participate in weekly lab meetings, journal clubs, seminars, and talks with outside speakers. **(Completed to date)**

Task 3: Meet with my mentor weekly to discuss ongoing experiments. **(Completed to date)**

Task 4: Review manuscripts related to my proposal as suggested by my mentor. **(Completed to date)**

Task 5: Complete all necessary lab work to fulfill the objectives outlined in the research proposal. **(In progress)**

Task 6: Complete coursework required by the Biological Sciences Ph.D. program. **(Completed)**

Task 7: Pass oral examination on the background of my research, present and successfully defend my research during the comprehensive preliminary/qualifying exam to pass onto Ph.D. candidacy. **(Completed)**

Task 8: Present my research at minimum of one national conference per year. **(Completed to date)**

Task 9: Write up experimental results in a timely manner for publication in peer-reviewed journals. **(Completed to date)**

Task 10: Collaborate with other students and investigators to fulfill my objectives. **(In progress)**

Task 11: Serve as a teaching assistant for two semesters. **(Completed)**

Task 12: Present a departmental seminar describing my completed thesis project, and defend my Ph.D. dissertation before my dissertation committee. **(In Progress)**

Task 13: Locate a suitable post-doctoral position for continuation of my training. **(In Progress)**

Milestones and Deliverables:

1. Completion of my preliminary/qualifying exam. **(Completed)**

2. Completion of required coursework to fulfill the Biological Sciences Ph.D. program. **(Completed)**

3. Complete two semesters as a teaching assistant. **(Completed)**

4. Present my first oral presentation at a national conference. **(Incomplete)**

5. Have my thesis research published in a well-respected, peer reviewed journal. **(Completed)**

6. Successfully defend my Ph.D. dissertation. **(In Progress)**

7. Obtain an appropriate and well-regarded post-doctoral position. **(In Progress)**

Task 1: Determine if Nrf2 regulates MDSC survival. (Completed)

Task 1A: Determine the rate of cell death of Nrf2^{-/-} MDSC compared to Nrf2^{+/+} MDSC. **(Completed)**

Task 1B: Determine if Nrf2 regulates GSH levels and MDSC apoptosis in response to oxidative stress.

Task 1C: To determine if GSH regulates apoptosis in MDSC.

Task 1D: Determine if tumor MDSC more susceptible to apoptosis than blood MDSC.

Task 1E: Determine if Nrf2 protects MDSC from the oxidative tumor microenvironment.

Task 1F: Determine if Nrf2 protects MDSC from hypoxia.

Outcomes/Products/Deliverables: Nrf2 decreases MDSC oxidative stress and enhances MDSC resistance to apoptosis. Nrf2 does not impact MDSC accumulation in tumor-bearing animals because Nrf2 deficiency enhances granulocytic MDSC accumulation.

Task 2: Determine if Nrf2 regulates tumor-bearer survival and MDSC suppressive activity.

Task 2A: Determine if Nrf2 regulates ROS, NO, and peroxynitrite production in MDSC. **(Completed)**

Task 2B: Determine if Nrf2 regulates the suppressive activity of MDSC. **(Completed)**

Task 2C: Determine if Nrf2 regulates MDSC accumulation and mammary tumor growth. **(Completed)**

Outcomes/Products/Deliverables: Nrf2 increases MDSC production of ROS, MDSC suppressive activity. Nrf2 does not impact primary tumor growth, but decreases survival of tumor-bearing mice.

Task 3: Determine if inhibition of MDSC sequestration of cysteine (via xCT) reduces MDSC accumulation, restores immune competence, delays metastatic disease, and increases survival time. (Completed)

Task 3A: Determine if SASP and NAC reduce MDSC production of ROS, NO, peroxynitrite, GSH levels, cystine transport, and reduce MDSC resistance to Fas-mediated apoptosis and suppressive activity. **(Completed)**

Task 3B: Determine if SASP and NAC affect MDSC accumulation and mammary tumor growth. **(Completed)**

Outcomes/Products/Deliverables: SASP reduces MDSC viability, GSH content, and cystine transport *in vitro*. There is no difference between inflammation-induced and conventional MDSC transport of cystine. SASP has no effect on tumor growth, metastatic disease, MDSC accumulation, or MDSC suppressive activity.

Reportable Outcomes

Milestones and Deliverables:

- Completed my preliminary/qualifying exam.
- Confirmed that Nrf2 decreases MDSC oxidative stress
- Confirmed that Nrf2 decreases MDSC apoptosis.
- Confirmed that Nrf2 does not impact MDSC accumulation in tumor-bearing animals.
- Confirmed that Nrf2 decreases MDSC differentiation from bone marrow progenitor cells.
- Confirmed that Nrf2 enhances MDSC suppressive activity.
- Confirmed that Nrf2 decreases survival of tumor-bearing mice.
- Have my research published in a peer reviewed journal.

Conclusions

- It has been demonstrated that Nrf2 regulates oxidative stress in MDSC and MDSC apoptosis. Research to ascertain which proteins downstream of Nrf2 which mediate MDSC oxidative stress and apoptosis would provide novel targets for future therapies aimed at reducing MDSC levels in tumor-bearing patients for enhancement of immunotherapeutic strategies of targeting cancer.
- It has been shown that Nrf2 does not increase MDSC accumulation in tumor-bearing animals but decreases MDSC differentiation from bone marrow progenitor cells. Research to ascertain which proteins downstream of Nrf2 which mediate MDSC differentiation would provide novel targets for future therapies aimed at reducing MDSC levels in tumor-bearing patients for enhancement of immunotherapeutic strategies of targeting cancer.
- Nrf2 does not impact primary tumor growth, but increases MDSC suppressive activity and reduces survival in tumor-bearing animals. Research to ascertain the mechanisms of Nrf2's pro-tumor activity and enhancement of MDSC suppressive activity would provide novel pathways to increase anti-tumor immunity.
- It has been demonstrated that inflammation enhances x_C - expression on MDSC, but higher x_C - expression does not enhance the ability of MDSC to transport cystine. *In vitro*, SASP inhibits cystine transport, reduces intracellular GSH, and increases cell death in MDSC. However, therapeutic administration of oral sulfasalazine to tumor-bearing animals has no effect on primary tumor growth, MDSC accumulation, metastatic disease, or MDSC suppressive activity. Therefore, SASP is a poor candidate for treatment of tumor-bearing individuals.

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Presentations:

- Daniel W. Beury, Phillip Fitzgerald, Suzanne Ostrand-Rosenberg "Does Glutathione Promote the Survival of MDSC by Detoxifying Endogenous Oxygen Radicals?" American Association of Immunologists 98th Annual Meeting. San-Francisco, CA. May 13-17, 2011 (poster presentation)

- Daniel W. Beury, Cassandra Nelson, Suzanne Ostrand-Rosenberg “Transcription Factor Nrf2 (NF-E2 Related Factor 2) Enhances Myeloid-Derived Suppressor Cell (MDSC) Accumulation and Tumor Progression” American Association of Immunologists 99th Annual Meeting. Boston, MA. May 4-8, 2012 (poster presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg “Communication among tumor-infiltrating immune cells enhances tumor-progression” UMBC Biological Sciences Departmental Seminar, Nov 28th, 2012 (oral presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg, “Anti-inflammatory effects of Myeloid-Derived Suppressor Cells and Macrophage crosstalk contribute to tumor progression” American Association for Cancer Research Annual Meeting. Washington, DC. April 6-10, 2013 (poster presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg, “Anti-inflammatory effects of Myeloid-Derived Suppressor Cells and Macrophage crosstalk contribute to tumor progression” American Association of Immunologists 100th Annual Meeting. Honolulu, Hawaii. May 3-7, 2013 (poster presentation)
- Daniel W. Beury, Katherine H. Parker, Maeva Nyandjo, Pratima Sinha, and Suzanne Ostrand-Rosenberg “Crosstalk between tumor cells, myeloid-derived suppressor cells, and macrophages differentially impacts their inflammatory capacity” Keystone Symposia Immune Evolution in Cancer. Whistler, British Columbia Canada. March 9-14 2014. (poster presentation)

Personnel receiving pay from the research effort:

- Daniel W Beury

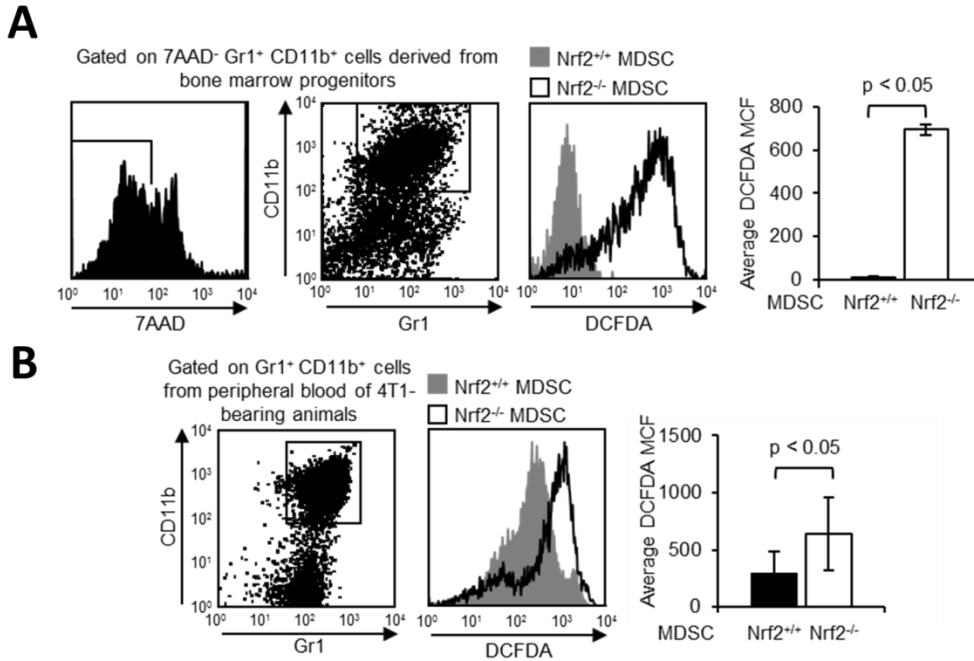
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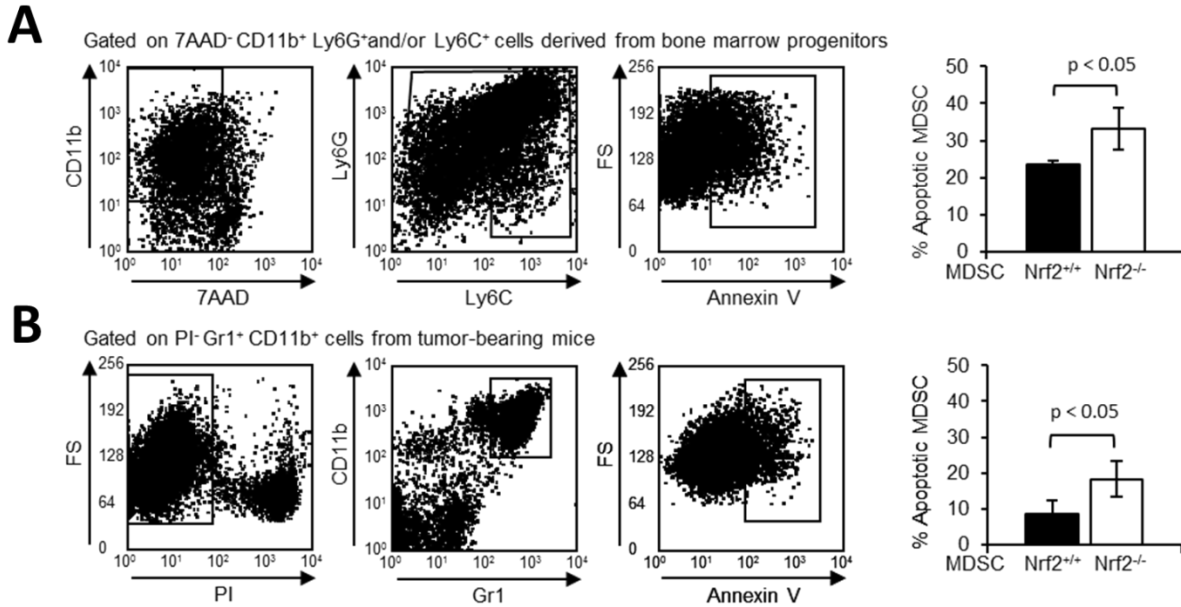
Supporting Data

Figure 1: Nrf2 decreases oxidative stress in vitro and in vivo



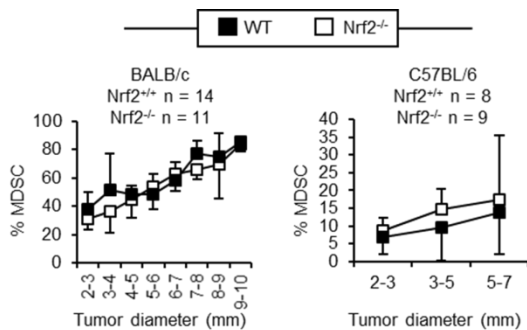
(A) Nrf2 decreases oxidative stress in vitro. Bone marrow cells from naïve BALB/c Nrf2^{+/+} or Nrf2^{-/-} mice were cultured for 4 days with 40ng/mL IL-6 and 40ng/mL GM-CSF, which drives the differentiation of bone marrow progenitor cells to MDSC. Cells were harvested and stained for live cells (7AAD⁻), the markers of MDSC (Gr1 and CD11b), and with DCFDA (measures intracellular ROS). 7AAD⁻ Gr1⁺ CD11b⁺ cells were gated and analyzed by flow cytometry. (B) Nrf2 decreases oxidative stress in vivo. Peripheral blood was harvested from BALB/c Nrf2^{+/+} and Nrf2^{-/-} mice bearing syngeneic 4T1 mammary carcinoma. Red blood cells were lysed and the remaining leukocyte fraction was stained and analyzed as in panel A. Nrf2^{+/+}: n=6; Nrf2^{-/-}: n=5. Gr1⁺ CD11b⁺ cells (MDSC) were gated and analyzed by flow cytometry for DCFDA. **(A and B)** Left histograms: representative staining of 7AAD, Gr1, CD11b, and DCFDA for individual mice. Right graphs: average MCF of DCFDA. Data were tested for statistical significance by Student's *t* test.

Figure 2: Nrf2 protects MDSC from apoptosis in vitro and in vivo



Bone marrow progenitor cell-derived (A) and 4T1 tumor-induced (B) MDSC were stained for Gr1, Ly6G, Ly6C, CD11b, and with Annexin V and propidium iodide (PI) or 7AAD, and analyzed by flow cytometry. Live Gr1⁺CD11b⁺ MDSC (PI⁻ or 7AAD⁻) were gated and assessed for Annexin V. (A and B) Left panels: representative staining of 7AAD, PI, Gr1, Ly6G, Ly6C, CD11b, and Annexin V. Right graphs: average percent of live Annexin V⁺Gr1⁺CD11b⁺ cells. For bone marrow MDSC, data represent one of two experiments with each experiment using one Nrf2^{+/+} and one Nrf2^{-/-} mouse. For 4T1-derived MDSC, Nrf2^{+/+}: n=6 and Nrf2^{-/-}: n=5. Data were tested for statistical significance using Student's *t* test.

Figure 3: Nrf2 does not impact the level of MDSC in tumor-bearing animals.



Nrf2^{+/+} and Nrf2^{-/-} mice on the BALB/c background (Left Panel) were injected with 4T1, and Nrf2^{+/+} and Nrf2^{-/-} mice on the C57BL/6 background (right panel) were injected with MC38 colon carcinoma. Tumor diameter was calculated as the average measurements of tumor length and width, and the percentage of MDSC in the leukocyte fraction from the peripheral blood was assessed by flow cytometry. MDSC percentages were plotted as a function of tumor diameter. Data were pooled from two independent experiments. BALB/c Nrf2^{+/+}: n= 6, BALB/c Nrf2^{-/-}: n=5, C57BL/6 Nrf2^{+/+} n=8, C57BL/6 Nrf2^{-/-} n= 9.

Figure 4: Nrf2 enhances the proliferation of granulocytic MDSC.

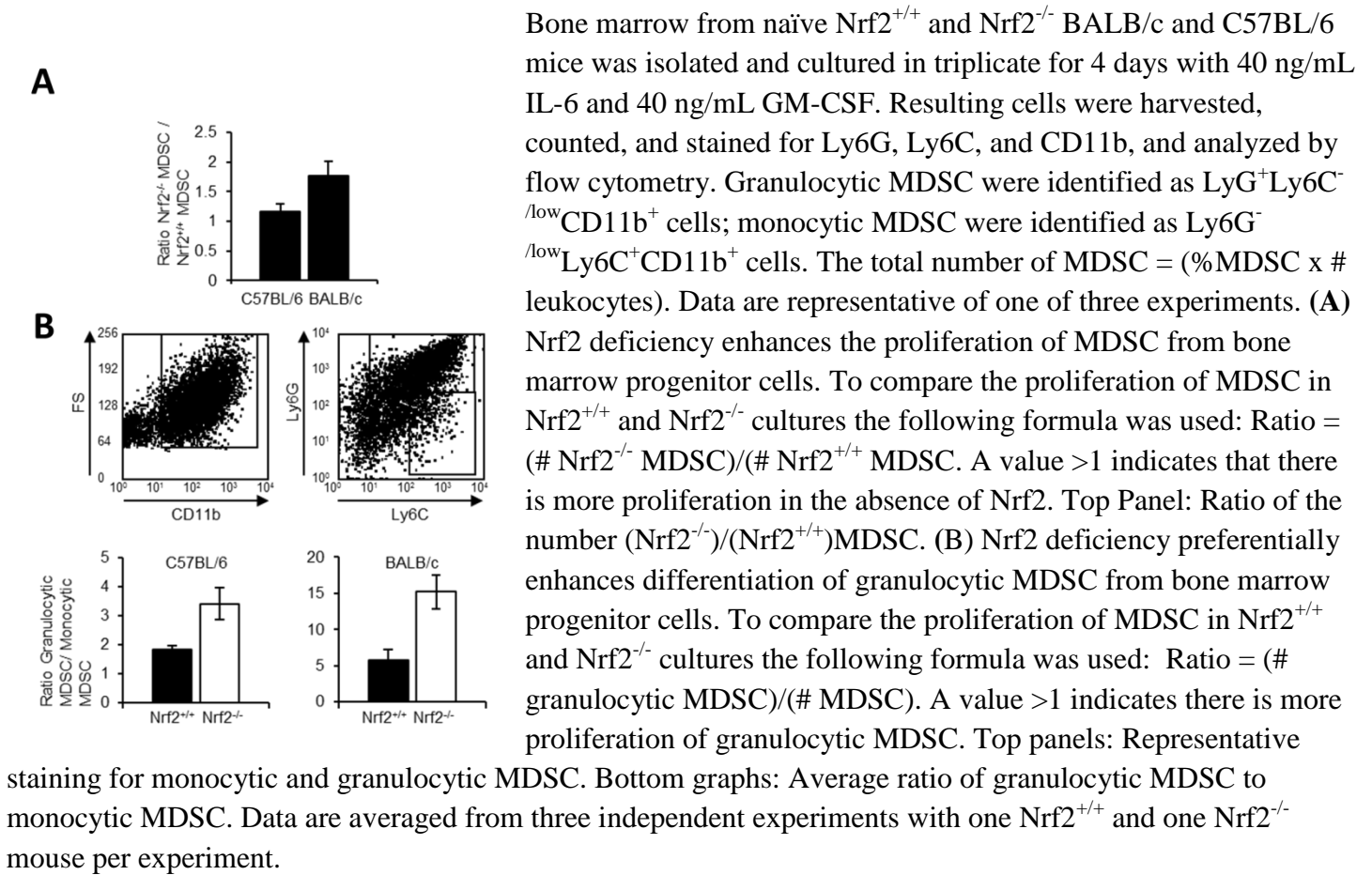
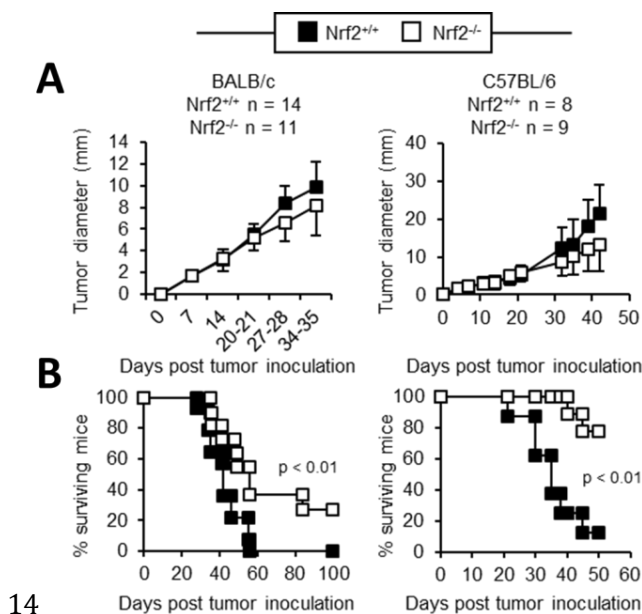


Figure 4: Nrf2 decreases survival time of tumor-bearing mice.



The mice of figure 3 were followed for primary tumor growth (A) and survival time (B). Data were pooled from two independent experiments. BALB/c Nrf2^{+/+}: n= 6, BALB/c Nrf2^{-/-}: n=5, C57BL/6 Nrf2^{+/+} n=8, C57BL/6 Nrf2^{-/-} n= 9. Survival time was tested for statistical significance by log-rank test.